OPTIMIZATION OF LIGNOCELLULOLYTIC ENZYME COCKTAIL PRODUCTION FROM *Cotylidia pannosa* AND ITS APPLICATION IN BIOETHANOL PRODUCTION AND DYE DECOLORIZATION

SYNOPSIS

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1. INTRODUCTION

The beginning of the third millennium has been and is still haunted by an increasing concern about the reliance of the human society on oil reserves and on the consequences for our planet as a whole from the rising carbon dioxide levels [1]. The sustained utilization of fossil fuels to meet the majority of the world's energy demand is endangered by increasing concentrations of CO_2 in the atmosphere and concerns over global warming [2]. The only justifiable alternate to address this issue is to employ carbon based sources that are everlastingly available in enormous amounts, and which can be used without the problem of greenhouse gas emission. Plant biomass is by far the lone carbon source that can fulfill these requirements: it arises by carbon dioxide fixation during photosynthesis, and its dry weight consists mainly of three polymers (cellulose, hemicelluloses, and lignin) whose monomer constituents (hexose and pentose sugars and phenylpropan compounds) can be transformed to useful starting materials for industry by fermentation or biotransformation (the so-called biorefinery concept) [1].

An imperative step in the notion of employing plant biomass as "biofuels/biorefineries" is the production of the monomeric components such as hexose and pentose sugars in a sufficiently high concentration by means of technologies that do not release harmful by-products. The only process that can meet this requirement in theory is enzymatic hydrolysis which has been studied since the early 1960s. These studies have shown that cellulolytic, hemicellulolytic, and ligninolytic enzymes are predominantly produced by fungi and some of these fungi have been successfully used for the production of enzymes used in the hydrolysis of plant cell wall material [1].

However, one of the biggest concerns with enzymatic hydrolysis is the price associated with the production of enzymes as well as inherent recalcitrance nature of plant biomass. The impact of enzyme costs to the economics of lignocellulosic biofuel production continues to be a hot topic for debate [3,4]. A solution to this problem is to find alternate ways for the production of enzymes which may be achieved by finding more potent microbial strains or by creating genetically modified strains that can excrete greater amounts of enzymes, or both [5].

Biofuels are solid, liquid or gaseous fuels obtained from freshly dead biological material as compared to fossil fuels, which are derived from long dead biological material. Various plants and plant-derived materials are used for biofuels manufacturing including grains (1^{st} generation) and lignocellulosic biomass (2^{nd} generation). The 'second generation' biofuels have the advantage of being cheap and abundant and do not compete with food crops [6]. Usually, the lignocellulosic biomass contains about 40−60% cellulose, 20−40% hemicelluloses, and 10−25% lignin [7]. Of all the components, the cell wall polysaccharides

can be hydrolysed into monomeric sugars which are used for biorefining to produce a range of bio-materials. Cellulose, hemicellulose and lignin, are not just individual units in a plant cell wall but are intimately interlocked making it extremely difficult to deconstruct enzymatically [8]. Lignin and carbohydrates (e.g., cellulose and hemicellulose) together form the lignin–carbohydrate complexes [9]. Anchoring of lignin to plant-wall polysaccharides contributes to recalcitrance [10, 11] by reducing the accessibility of cellulose to enzymes [12]. For complete deconstruction of these heterogeneous structures in the plant cell wall synergistic reactions of enzymes, such as cellulases, hemicellulases, accessory enzymes and lignin-modifying enzymes is required [13].

Fungi and bacteria both have been profoundly exploited for their ability to hydrolyze lignocellulosic materials by producing a wide variety of cellulases and hemicellulases [14]. To date, the majority of enzymes developed and being tested for lignocellulose degradation are from fungi [15] because of their ability to produce profuse amounts of cellulases and hemicellulases secreted directly into the medium for easy extraction and purification. Various wood-rot fungi like white- and brown-rot, have been reported to effectively degrade lignin, cellulose and hemicellulose. They produce extracellular enzymes like ligninase, cellulase and hemicellulase to degrade the lignocellulosic complex [16]. The lignin-degrading enzymes secreted by white-rot fungi enable them to completely mineralize lignin to carbon dioxide and water, in turn exposing the hemicellulose and cellulose in the wood matrix [17] which are hydrolyzed by conglomerates of hemicellulase and cellulase. White rot fungi are considered as the major lignin degrader [18]. White-rot basidiomycetes such as *Pleurotus ostreatus, Trametes versicolor*, *Phanerochaete chrysosporium*, *Ganoderma lucidum*, *Coriolus versicolor* and *Polyporus brumalis,* constitute a crucial source of organisms with lignocellulosic machinery for the production of extracellular ligninolytic (laccase) and hydrolytic (cellulases and hemicellulases) enzymes, which are responsible for the degradation of major substrate components of lignocellulosic biomass into value added products [19]. The need for developing more efficient enzyme preparations arises for the enzymatic saccharification process to be more economical. This necessitates the isolation and screening of novel fungi capable of efficient degradation of lignocellulosic biomass by employing a proficient lignocellulolytic enzyme system. In this context, the fauna of North-Western Himalayan region of India still needs to be explored extensively and as such the prospective for discovering novel strains capable of producing hydrolytic and ligninolytic enzymes of industrial potential exists [20] which is being investigated in the present study.

2. OBJECTIVES OF THE STUDY

Understanding the importance and the necessity of identifying novel fungal strains capable of efficient lignocellulosic biomass degradation by engaging their proficient lignocellulolytic enzyme system, the following objectives were laid down for the present study:

Objective 1:

Screening and identification of fungal cultures for lignocellulolytic enzyme activities

Objective 2:

Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged and solid state fermentation using one factor at a time approach

Objective 3:

Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged fermentation by using Response Surface Methodology (Multifactorial approach)

Objective 4:

Characterization of crude enzyme cocktail for lignocellulolytic enzyme activities and its application in saccharification of wheat bran for bioethanol production and dye decolorization

OBJECTIVE 1

2.1 Screening and identification of fungal cultures for lignocellulolytic enzyme activities.

One of the biggest concerns with enzymatic hydrolysis is the price associated with the production of enzymes. A solution to this problem is to find alternate ways for the production of enzymes which may be achieved by finding more potent microbial strains or by creating genetically modified strains that can excrete greater amounts of enzymes, or both.

This necessitates the isolation and screening of novel fungi capable of efficient degradation of lignocellulosic biomass by employing a proficient lignocellulolytic enzyme system. Therefore, in the present study 19 fungal strains belonging to North-Western Himalayan region were screened with the objective of finding a novel fungal strain having the potential of being employed in the efficient degradation of lignocellulose biomass.

MATERIALS AND METHODS

Culture collection

A collection of 19 fungal strains identified by fruiting bodies and morphological characteristics were procured from Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur, India. All the cultures were collected from different geographical regions of North-Western Himalayan ranges in Himachal Pradesh. The yeast culture of *Saccharomyces cerevisiae* MTCC 174 and standard fungal culture of *Trichoderma reesei* MTCC 164 were procured from Institute of Microbial Technology (IMTech), Chandigarh, India. These isolates were maintained on yeast extract peptone dextrose (YEPD) agar at 4°C.

Screening of fungal cultures for lignocellulolytic enzyme activities

19 fungal isolates were screened qualitatively by agar plate assay and quantitatively by spectrophotometric method for cellulase and xylanase enzyme activity using carboxymethyl cellulose (CMC) and birchwood xylan respectively as standards according to the method of Mandels et al., (2010) [21]. Laccase activity was determined by the method of ABTS oxidation as given by Bourbonnais et al., (1998) [22]**.** One unit of enzyme activity was defined as an amount of enzyme that releases one micromoles of product per minute under standard reaction conditions.

Identification of the potential fungal strain

Morphological characteristics

Cultural characteristics such as colony appearances, mycelial textures on potato dextrose agar plates were observed after 3–7 days of incubation under the standard incubation conditions. Growth rate on PDA via colony diameter was also measured. For microscopic observation, mycelial plugs of *C. pannosa* were transferred onto lactophenol cotton blue stain and examined the preparation under low and high, magnification for the presence of characteristic mycelia and spores.

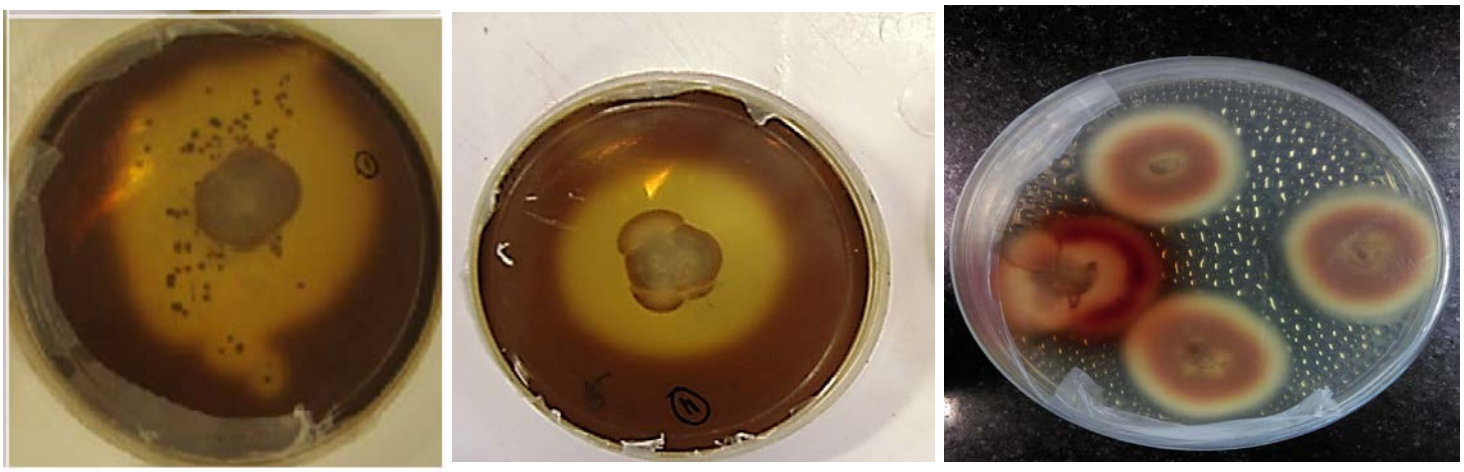
Molecular identification using Internal transcribed spacer (ITS) region

The fungus was inoculated on potato dextrose agar (PDA) for one week and then transferred into potato dextrose broth (PDB) at 30°C for 7 days. The fungal mycelium was filtered, rinsed with sterilized water, frozen, and grounded into fine powder with pre-chilled mortar and pestle. The genomic DNA was extracted using CTAB method [23] with some modification. The fungus was identified by targeting Internal transcribed spacer (ITS) region: ITS1 and ITS4 [24]. The PCR amplification was carried out using PCR conditions of 1 cycle at 94°C for 5 min; 30 cycles (95°C for 45 s; 53°C for 1 min; 72°C for 2.5 min); 1 cycle at 72°C for10 min; hold at 4°C in thermocycler (Bio-Rad T100). The PCR products were then analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1X Tris-acetate-EDTA buffer with 0.5 mg/ml of ethidium bromide at a constant voltage of 80 V for 45 min, then visualized under UV light to confirm the presence of the amplified DNA. The amplified PCR fragments were purified with DNA purification kit (Real Biotech Corporation), following the manufacturer's instruction and then sequenced. The ITS regions were employed to search the closest sequences from the GenBank database (http://www.ncbi.nlm.nih.gov) using a BLAST search [25] to clarify the generic and species level of the fungal isolate. DNA sequences were multiple aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [26]. Phylogenetic analysis was carried out on multiple sequence alignment in order to construct phylogenetic trees by UPGMA method. Bootstrap confidence values were generated using 100 permutations of the data set to derive the nucleotide sequence similarities.

RESULTS AND DISCUSSION

Screening of fungal cultures for lignocellulolytic enzyme activities

Both quantitative and qualitative assays revealed that out of the 19 fungal cultures screened for lignocellulolytic enzyme production, *C. pannosa* was the most efficient producer of lignocellulolytic enzymes. Qualitative assay based on agar plate method (**Figure 1**) showed that *C. pannosa* formed an 80 mm zone of clearance on CMC containing agar plate (cellulase activity), a 50 mm zone of clearance on birchwood xylan containing agar plate (xylanase activity) and a brown colouration on guaiacol (0.25mM) containing agar plate (Laccase activity).

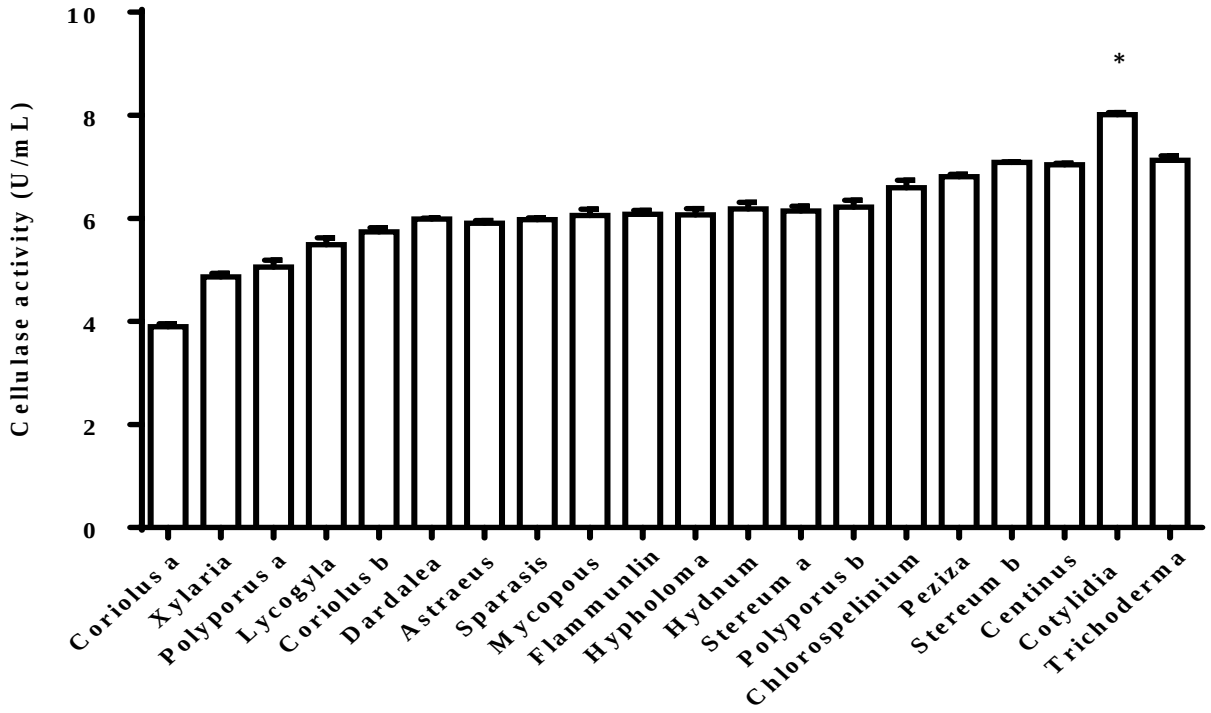


(a) **(b)** **(c)**

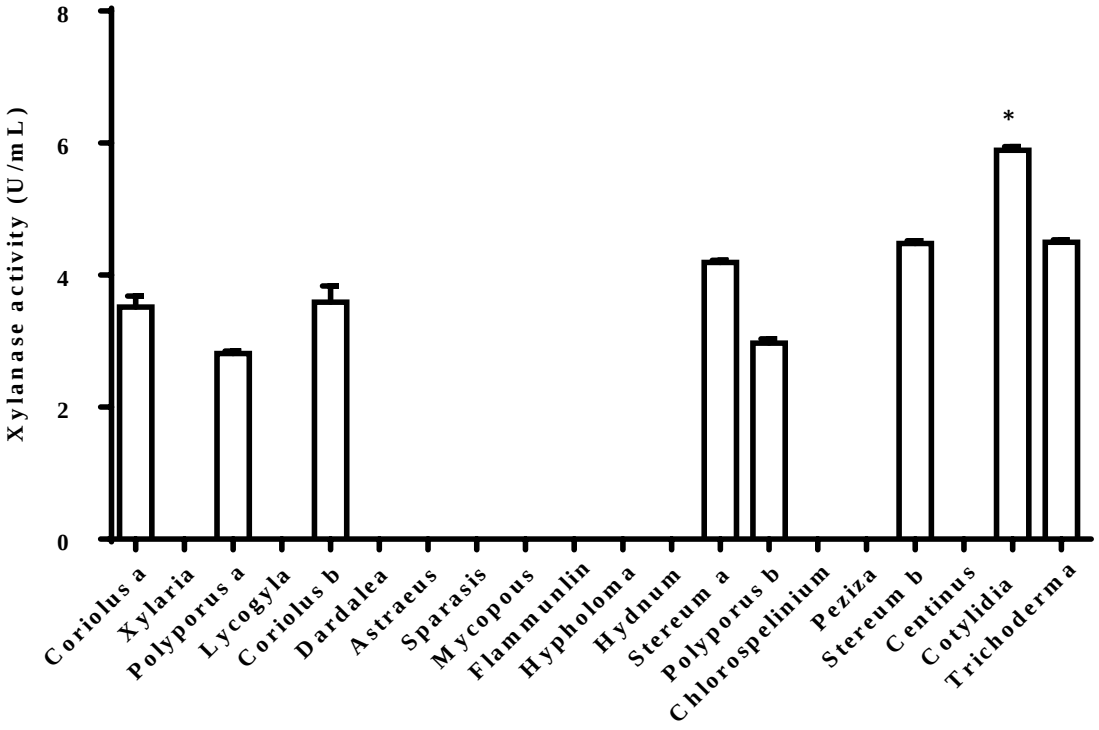
Figure 1: *Cotylidia pannosa* exhibits the presence of (**a)** cellulase (80 mm zone), **(b)** xylanase (50 mm zone) and **(c)** laccase enzymes activities.

The quantitative assay based on spectrophotometric method (**Figure 2**) also indicated that *C. pannosa* possessed maximum cellulase (8.01 U/ml), xylanase (5.89 U/ml) and laccase (5.01 U/ml) activity.

(a)


Cellulase activity (U/mL)
10
8
6
4
2
0
*
Coriolus a
Xylaria
Polyporus a
Lycogyla
Coriolus b
Dardalea
Astraeus
Sparasis
Mycopous
Flammunlin
Hypholoma
Hydnum
Stereum a
Polyporus b
Chlorospelinium
Peziza
Stereum b
Centinus
Cotylidia
Trichoderma

(b)


Xylanase activity (U/mL)
8
6
4
2
0
*
Coriolus a
Xylaria
Polyporus a
Lycogyla
Coriolus b
Dardalea
Astraeus
Sparasis
Mycopous
Flammunlin
Hypholoma
Hydnum
Stereum a
Polyporus b
Chlorospelinium
Peziza
Stereum b
Centinus
Cotylidia
Trichoderma

(c)

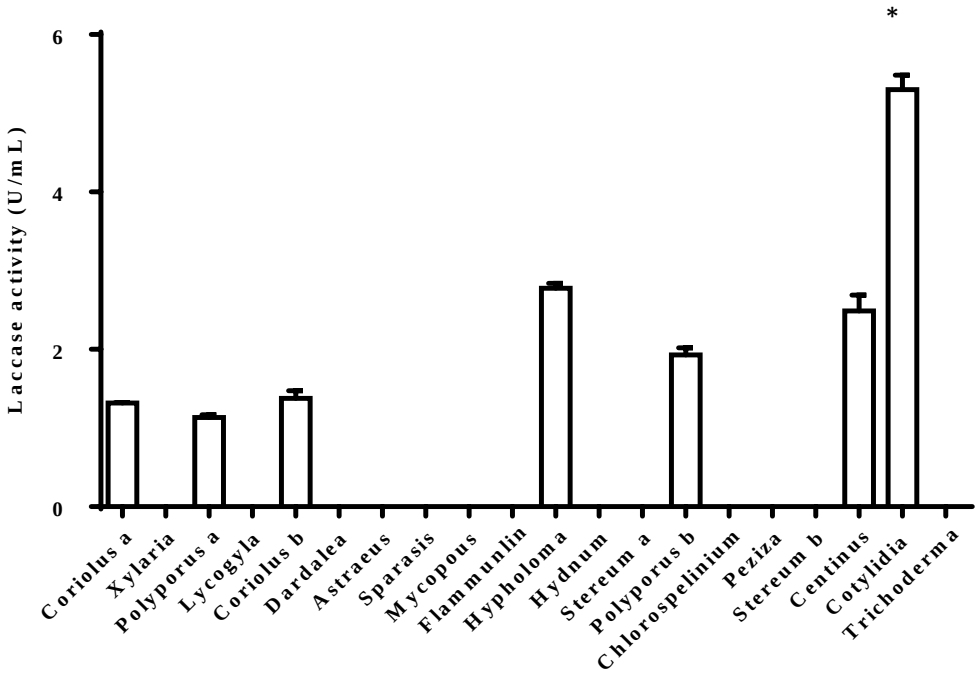


Figure 2: Screening of different fungal cultures for **(a)** cellulase activity, **(b)** xylanase activity and **(c)** laccase activity

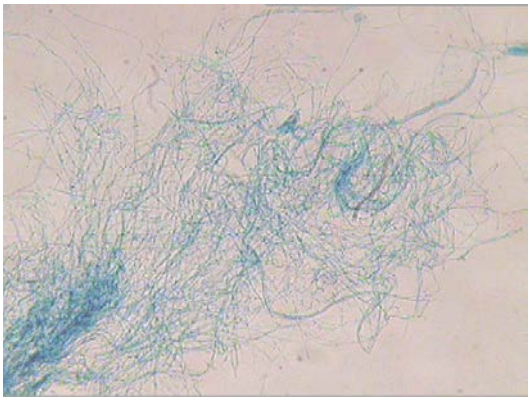
Identification of most potential fungal strain

Morphological characteristics

Cotylidia is a fungal genus characterized by ellipsoidal shaped spore formation, aseptate hyphae and the absence of clamp connections (**Figure 3)**.

(a)

(b)



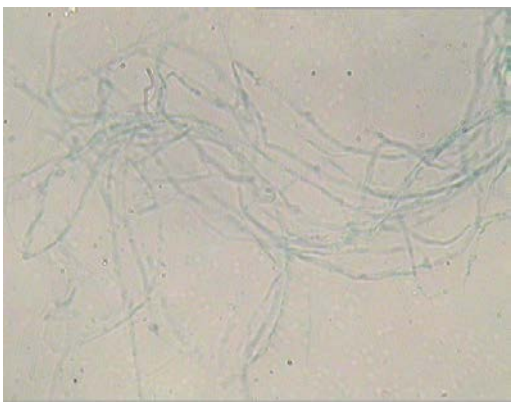


Figure 3: Morphological characteristics of *Cotylidia pannosa* (a) Mycelium morphology of *C. pannosa* on PDA plate and (b) Microscopic observation of hyphae of *C. pannosa* (10 x, 40 x)

Molecular identification using Internal transcribed spacer (ITS) region

A phylogenetic tree was constructed from a dataset consisting of 50 sequences aligned with fungal strain. Our fungal strain identified as *Cotylidia pannosa* formed a clade with *Phodoscypha petalodes* isolate DK09 sequence (AM773629) with an alignment score of 96.93%, based on Clustal analysis and a nucleotide similarity of 96% based on BLASTn analysis.

CONCLUSION

This study identified the potential of *C. pannosa* (Gene Bank Accession No. KT008117), a white rot fungus as an efficient producer of lignocellulolytic enzyme repertoire. Out of the 19 fungal strains that were screened for the presence of an efficient lignocellulolytic enzyme system, *C. pannosa* was found to be the most proficient producer of lignocellulolytic enzymes and hence was selected for further optimization studies.

OBJECTIVE 2

2.2 Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged and solid state fermentation by using one factor at a time approach.

Based on the observation and results derived from objective 1, *C. pannosa* was selected for further studies with the view of optimizing various parameters related to lignocellulolytic enzymes production. In this objective various parameters such as temperature, incubation time and pH were investigated to identify the optimum conditions supporting maximum lignocellulolytic enzyme activities.

MATERIALS AND METHODS

Lignocellulosic substrate: Wheat bran

Wheat bran was used as lignocellulosic substrate for screening of lignocellulolytic activities. The cellulose and hemicelluloses content in the wheat bran was determined by the method of Crampton and Maynard [27]; while lignin content was estimated by the method of Goering and Van Soest [28].

Effect of saccharification parameters

Submerged fermentation (SmF)

A 2.0 g of wheat bran was added to 100 ml of growth medium (YEP) consisting of 1% yeast extract and 2% peptone followed by sterilization at 121°C for 15 min at 15 psi. A spore inoculum of *C. pannosa* (2.9×10^8 fungal spores /ml) was inoculated aseptically in 100 ml of sterilized growth medium followed by incubation for 96 h at 30°C. A 5 ml aliquot of hydrolyzed biomass was withdrawn from the flask after a regular interval of 12 h upto 96 h and centrifuged at 4°C at 7000g for 15 min. The supernatant collected was analyzed for cellulase, xylanase enzyme activities by estimating the amount of reducing sugars produced using dinitrosalicylic acid method [29] and laccase activity by measuring the oxidation of ABTS indicated by an increase in absorbance at 420 nm. The lignocellulolytic enzyme production from *C. pannosa* using the most potent substrate was optimized at different fermentation parameters such as temperature (25 – 40°C), incubation time (0 – 108 h) and pH (3.0 – 6.0).

Solid State Fermentation (SSF)

For solid state fermentation, a spore inoculum of *Cotylidia pannosa* (2.9×10^8 fungal spores/mL) was inoculated in 2 g of different substrates moistened with growth media 100% moisture level. Subsequently the flasks were incubated at variable growth parameters i.e temperature (25 – 42°C), time (0 – 108 h) and pH (3.0 – 6.0). After incubating the flasks

under static conditions, enzyme was extracted using sodium acetate and phosphate buffer (pH 5) by vortexing each flask for 15 minutes. After proper mixing had been done the crude enzyme was filtered using a muslin cloth and subjected to centrifugation at 7,000g for 20 minutes. The supernatant thus obtained contained crude enzyme and hence was subjected to enzyme activity analysis as described in the submerged fermentation section.

RESULTS AND DISCUSSION

Effect of saccharification parameters

Submerged fermentation

In order to establish a suitable application in saccharification of lignocellulosic wastes, the production of lignocellulolytic enzyme by *C. pannosa* was optimized under submerged fermentation conditions using wheat bran as the carbon source. The maximum lignocellulolytic activity was detected at pH of 5.0 (**Figure 4**) and at a temperature of 30°C (**Figure 5**).

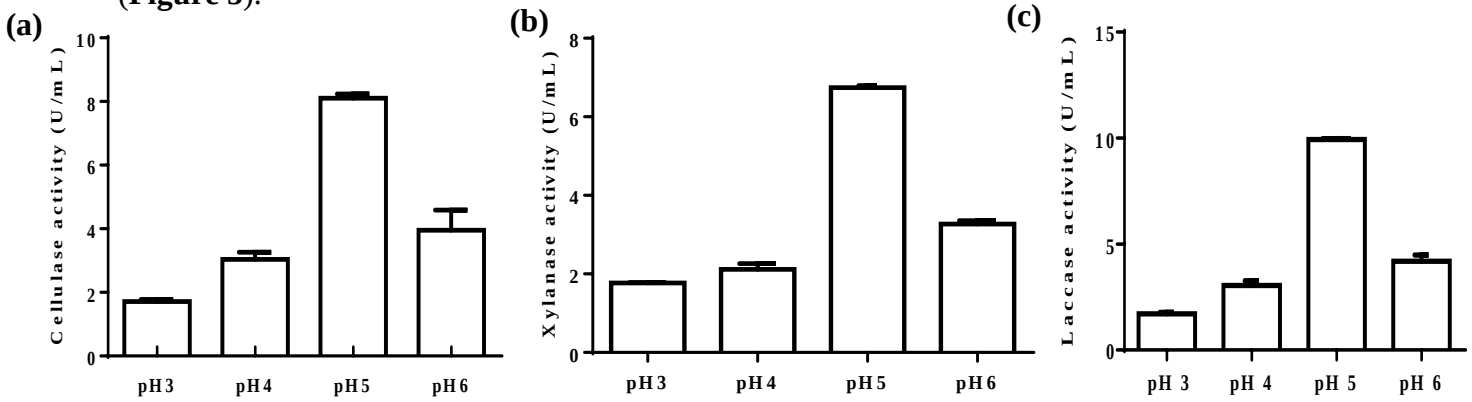


Figure 4: Effect of pH on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *Cotylidia pannosa* in submerged fermentation

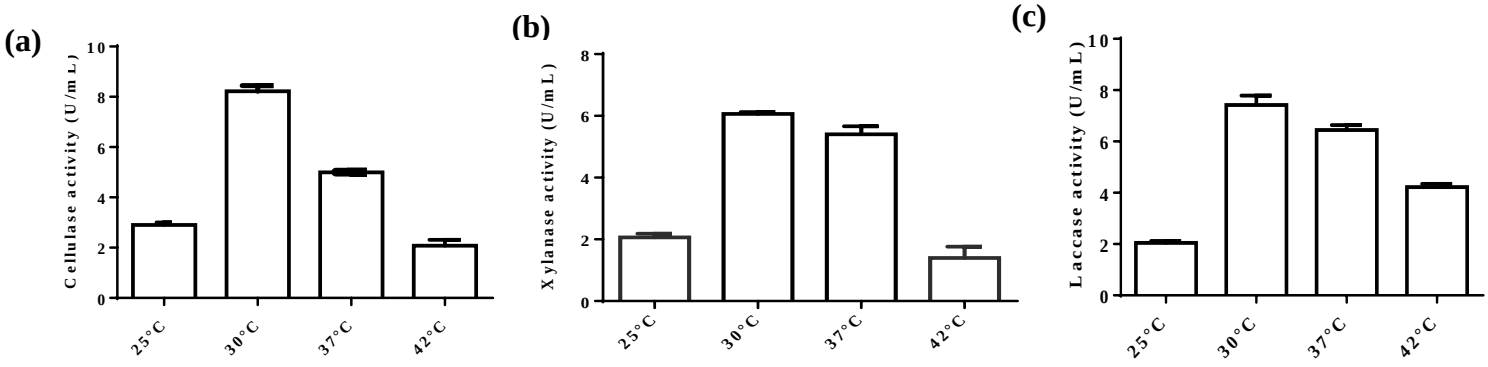


Figure 5: Effect of temperature **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *Cotylidia pannosa* in submerged fermentation

As shown in **Figure 6**, *C. pannosa* showed maximum cellulase activity of 8.44 U /ml after an incubation time of 56 h, xylanase activity of 5.0 U/ ml and laccase activity of 7.23 U/ml after 72 h.

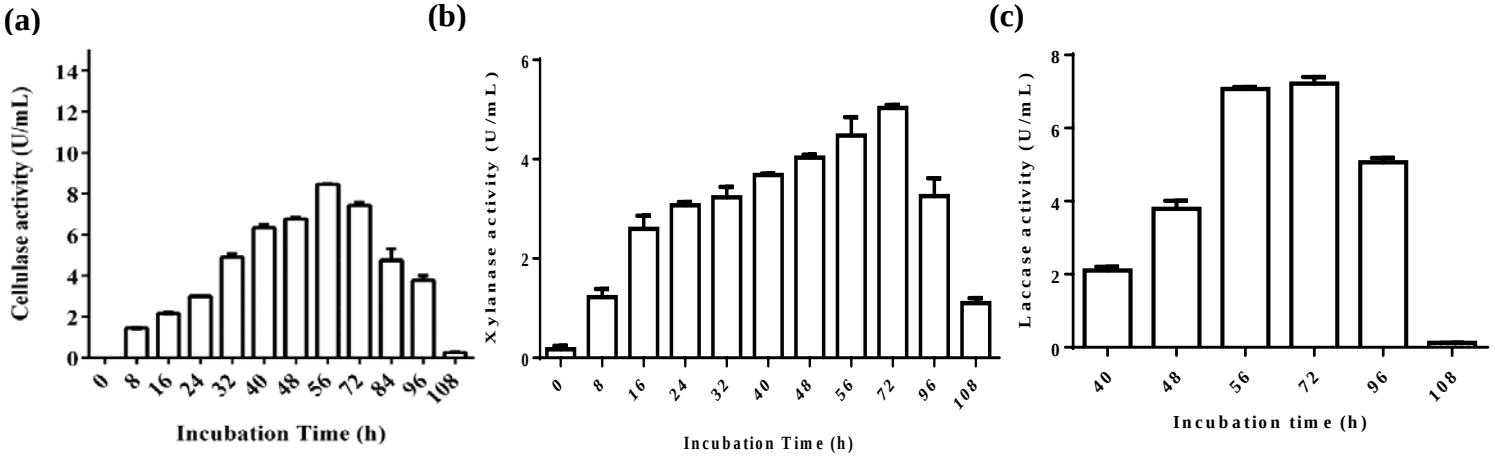


Figure 6: Effect of incubation time on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *Cotylidia pannosa* in submerged fermentation

Solid State Fermentation

Solid state fermentation using wheat bran as a substrate also revealed it as the potential substrate, although the lignocellulolytic activity obtained was less as compared to that obtained under submerged fermentation. Lignocellulolytic enzyme production by *Cotylidia pannosa* was optimized under solid state fermentation conditions using wheat bran as the carbon source. The maximum lignocellulolytic activity was detected at pH of 5.0 (**Figure 7**) and at a temperature of 30°C (**Figure 8**).

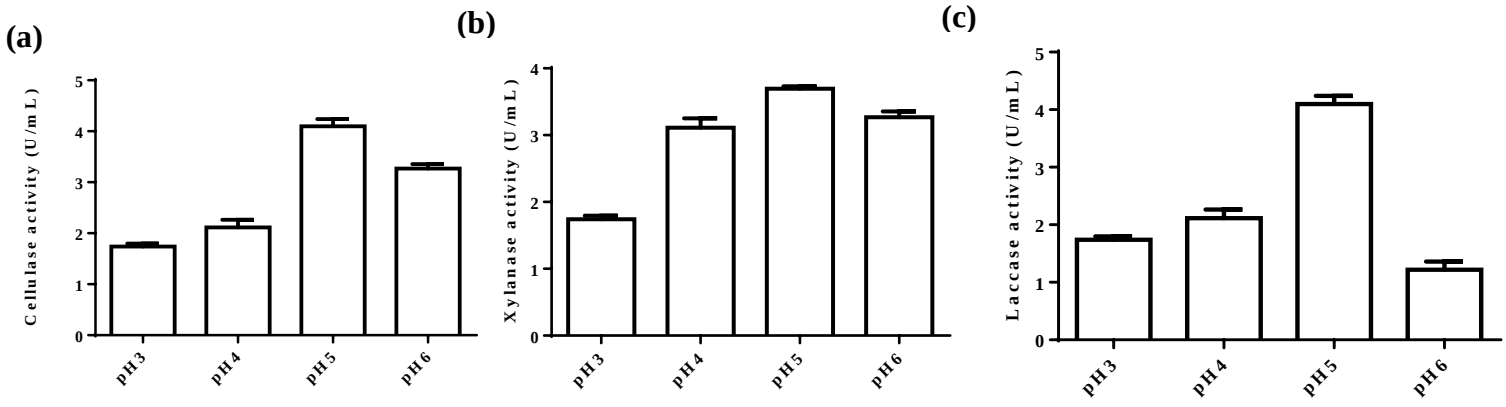


Figure 7: Effect of pH on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *Cotylidia pannosa* in solid fermentation

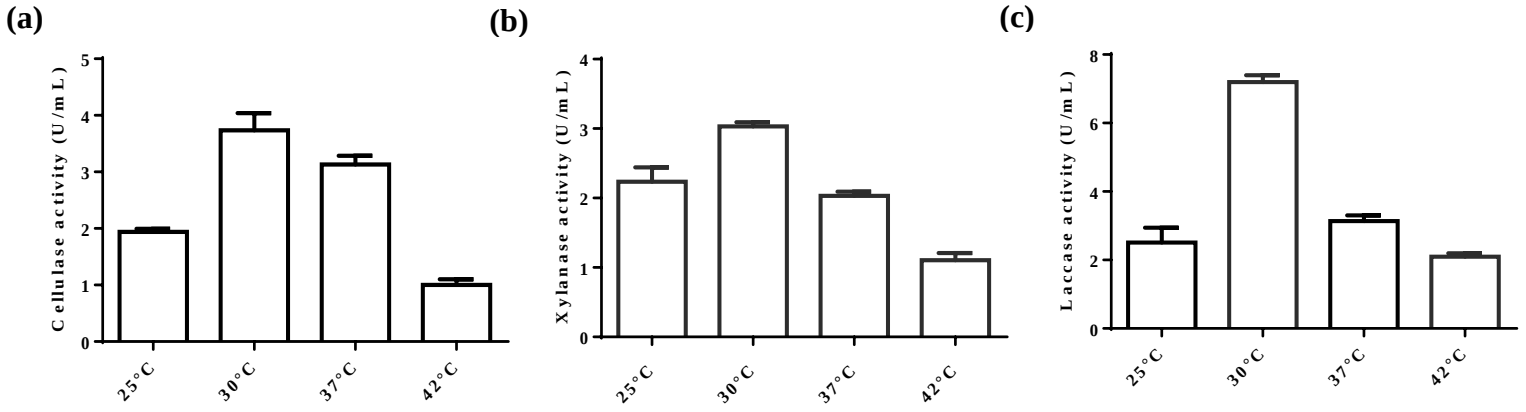


Figure 8: Effect of temperature on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *Cotylidia pannosa* in solid fermentation

As shown in **Figure 9**, *C. pannosa* showed maximum cellulase activity of 4.33 U /ml after an incubation time of 96 h, xylanase activity of 5.23 U/ ml after an incubation time 56 h and laccase activity 5.22 U/ml after 72 h.

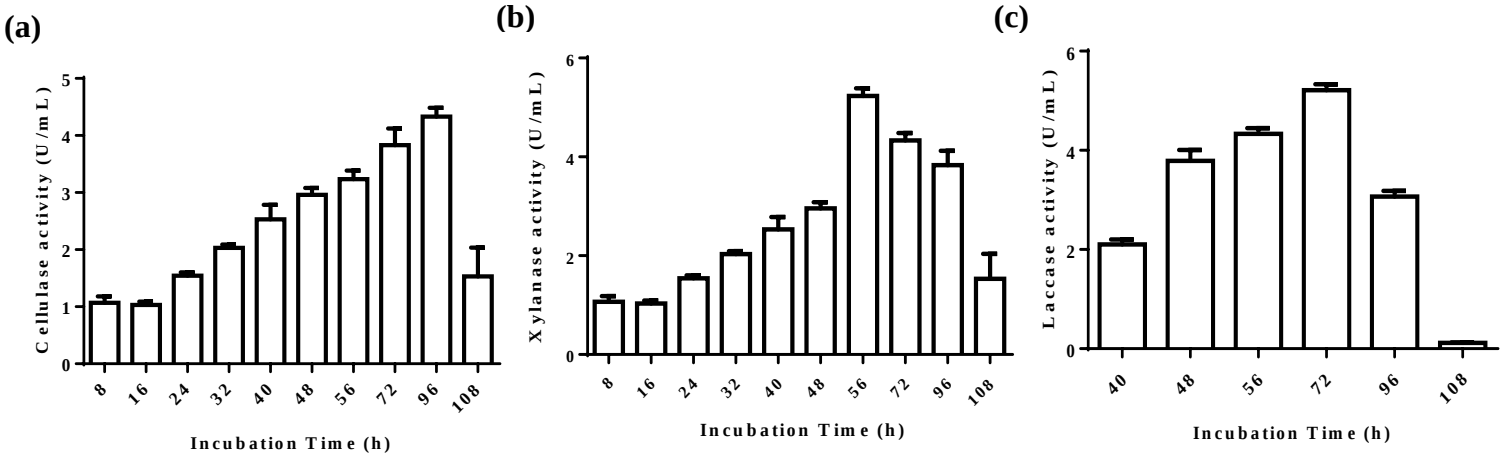


Figure 9: Effect of incubation time on **(a)** cellulase **(b)** xylanase and **(c)** laccase activity of *Cotylidia pannosa* in solid fermentation

It has been observed that pH higher or lower than the optimum pH causes a decrease in the production of lignocellulolytic enzyme primarily because of the fact that lignocellulolytic enzyme production by mould cultures is known to be very sensitive to pH [30]. As the temperature was increased to 30°C there was an increase in enzyme activity followed by a decrease in activity as the temperature was increased beyond the optimum level. The cellulolytic activity decreases on increase in temperature as the high temperature of the

medium is reported to change the membrane composition and cause protein catabolism and inhibition of fungal growth. A short optimum incubation time for cellulolytic activity is preferable because high amount of enzyme activity can be achieved in lesser time. In various other studies, the maximum enzyme production was achieved after a longer incubation time period as observed for our strain *C. pannosa*.

CONCLUSION

From this objective, it was observed that *C. pannosa* performs more efficiently under conditions of submerged fermentation as compared to solid state fermentation with maximum cellulase, xylanase and laccase activity at pH of 5.0 and at a temperature of 30°C when incubated for 56 h.

OBJECTIVE 3

2.3 Optimization of lignocellulolytic enzyme activities by *Cotylidia pannosa* under submerged using Response Surface Methodology (Multifactorial approach).

The bioconversion of lignocelluloses relies heavily on major technological innovations centered on efficient process design for production of enzymes. Response surface methodology (RSM) is a well-practiced one for creation of a process model in the form of a non-linear regression equation by considering the effect of individual, square and interaction terms of process variables on the ouput [31]. Moreover, the response optimizer function of RSM is helpful for predicting the process variables for getting optimum output. A white rot fungus *Cotylidia pannosa* reported in our recent work, possesses cellulase and xylanase activities along with the laccase activity [20]. The production of lignocellulolytic enzymes through submerged fermentation is mainly dependent on process variables namely temperature (°C), pH, incubation time (h) and agitation rate. Therefore, the present study utilized RSM for modeling and optimization of lignocellulosic enzymes production by *Cotylidia pannosa* through submerged fermentation using wheat bran as a substrate.

MATERIALS AND METHODS

Microbial culture and maintenance

The culture of *C. pannosa* (Gene Bank Accession No. KT008117) obtained from Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur, India was maintained as mentioned in objective 2.

Modelling and optimization of lignocellulolytic enzyme production through RSM

For enzyme production, 2% wheat bran was added to the YEP culture media consisting of 1% yeast extract and 2% peptone followed by sterilization at 121°C for 15 min at 15 psi. A spore inoculum of *C. pannosa* (2.9×10^8 fungal spores/mL) was inoculated aseptically into 100 mL of sterilized growth medium. Based on the Central Composite Design (CCD) of RSM, four independent variables, including temperature (A, 25 − 40°C), pH (B, 4 − 6), incubation time (C, 24 − 120 h), and agitation rate (50 − 150 rpm) were studied at four different levels, based on preliminary results on lignocellulolytic enzyme production under submerged fermentation using one factor at a time approach. An aliquot of hydrolyzed broth was withdrawn from the flask after a regular interval and centrifuged at 4°C at 7000g for 15 min. The supernatant after centrifugation was collected and used for determination of cellulase, xylanase and laccase enzyme activities.

Non-linear regression analysis was carried out based on the data collected as per CCD planning for responses, namely cellulase, xylanase and laccase activity using MINITAB 16 software which resulted in a second-order polynomial equation. The coefficient of the non-linear regression model can be determined using the method of least squares. The effect of the parameters and their interaction terms on the response has been studied by conducting the significance tests and Analysis of variance (ANOVA) has been carried out on each response to check the adequacy of the model. The detailed analysis of the effect of parameters and their interactions on the response was also done through the surface plots using MINITAB 16 software. The optimized variables for the higher cellulase, xylanase and laccase activities (U/ml) have been chosen through the response optimizer function of the MINITAB 16 software.

RESULTS AND DISCUSSION

Modelling and optimization of lignocellulolytic enzyme production through RSM

Temperature, pH, incubation time and agitation rate are the critical factors in Smf and their importance in enzyme production has been well established. One-factor-at-a-time approach was used to identify the concentration levels of these parameters. The process variables of SmF i.e., temperature (Temp), pH, incubation time (IT) and agitation rate (Agtn) have been selected as input variables and experiments have been executed based on CCD for developing a second order polynomial response surface model for lignocellulolytic enzyme production by *C. pannosa*. These experiments were performed in triplicates and cellulase, xylanase and laccase activity of *C. pannosa* have been expressed as a non-linear function of the input process parameters as follows:

Cellulase (U/ml) = -121.7 + 6.948 Temp (^oC) + 6.15 pH + 0.4592 IT (h) - 0.0775 Agtn (rpm) - 0.1152 Temp (^oC)*Temp (^oC) - 0.389 pH*pH - 0.002070 IT (h)*IT (h) + 0.001302 Agtn (rpm)*Agtn (rpm) + 0.0505 Temp (^oC)*pH - 0.000577 Temp (^oC)*IT (h) - 0.000537 Temp (^oC)*Agtn (rpm) - 0.01651 pH*IT (h) - 0.02357 pH*Agtn (rpm) 0.000262 IT (h) *Agtn (rpm)

Xylanase (U/ml) = -141.3 + 7.341 Temp (^oC) + 12.89 pH + 0.4048 IT (h) - 0.1069 Agtn (rpm) - 0.1279 Temp (^oC)*Temp (^oC) - 1.907 pH*pH - 0.001635 IT (h)*IT (h) + 0.001002 Agtnn (rpm)*Agtn (rpm) + 0.2178 Temp (^oC)*pH - 0.001424 Temp (^oC)*IT (h) - 0.001204 Temp (^oC)*Agtn (rpm) - 0.01705 pH*IT (h) - 0.01074 pH*Agtn (rpm) 0.000014 IT (h)*Agtn (rpm)

Laccase (U/ml) = -116.8 + 8.081 Temp (^oC) - 3.98 pH + 0.3824 IT (h) - 0.1490 Agtn (rpm) 0.1251 Temp (^oC)*Temp (^oC) + 0.319 pH*pH - 0.002497 IT (h)*IT (h) + 0.000625 Agtn (rpm)*Agtn (rpm) + 0.0109 Temp (^oC)*pH - 0.000331 Temp (^oC)*IT (h) - 0.000015 Temp

(^{o}C)*Agtn (rpm) - 0.00222 pH*IT (h) + 0.00583 pH*Agtn (rpm) + 0.000142 IT (h)*Agtn (rpm)

The predictive ability of developed non-linear regression models was further confirmed through significance test and ANOVA tests. In case of cellulase, individual, square and interaction effects of SmF variables seemed to have significant effect (P<0.05) except the individual, square and interaction effects of pH. In case of xylanase, except the individual and interaction effects of agitation, the individual, square and interaction effects of other SmF variables (Incubation time, temp and pH) determined significant impact (P<0.05). Whereas the important factors for determining the laccase activity included only the individual and square effects of incubation time and agitation. From the results of ANOVA test, significant contributors towards cellulase activity were observed to be the linear, squared, and interaction terms of temp, pH and agitation. In case of xylanase activity, the linear, square and interaction effects of temp, incubation time, pH were also observed to play significant role. The most significant contributions for laccase activity included the linear, square and interaction effects of incubation time and agitation.

The fitness and adequacy of the developed non-regression model was further confirmed through the R^2 and adjusted R^2 values (Cellulase: R^2-98.20%, Adj. R^2 - 96.63%; Xylanase: R^2-98.14%, Adj. R^2 - 96.503%; Laccase: R^2-98.45%, Adj. R^2 - 97.10%). Further, the interaction effects of variables selected on production of enzymes were studied by plotting three dimensional surface curves to determine the optimum level of each variable for maximum enzyme activity.

The interaction effects of SmF variables on cellulase activity are depicted in response surface plots from **Figure 10 (a − c)**. **Figure 10 (a)** indicated that the higher cellulase activities were observed at intermediate values of temperature and pH. Later on, irrespective of increase or decrease in pH and temperature conditions, no significant effect on cellulase production was observed. The pH of the substrate plays a critical role in production of lignocellulolytic enzymes by affecting either the microbial growth or denaturating the enzymes. The change in pH is also reported to affect the transport of various components across the cell membrane and denaturation of the enzyme activities. **Figure 10 (b)** showed that the increase in temperature with incubation time resulted in higher cellulase production with a maximum cellulase production at 32°C in 72 h. Usually, the optimum temperatures and incubation time for lignocellulolytic enzymes production vary with the use of different strains. The cumulative effect of pH and incubation time on cellulase production (**Figure 10 (c)**) revealed that the higher cellulase production was mainly recorded in the mid values of pH and incubation time with decreased trend after pH of 5.5 and prolonged incubation time of 90 h.

This type of behaviour is mainly attributed to the inefficient transport across membranes at higher pH conditions and denaturation of cellulase activity at longer incubation times.

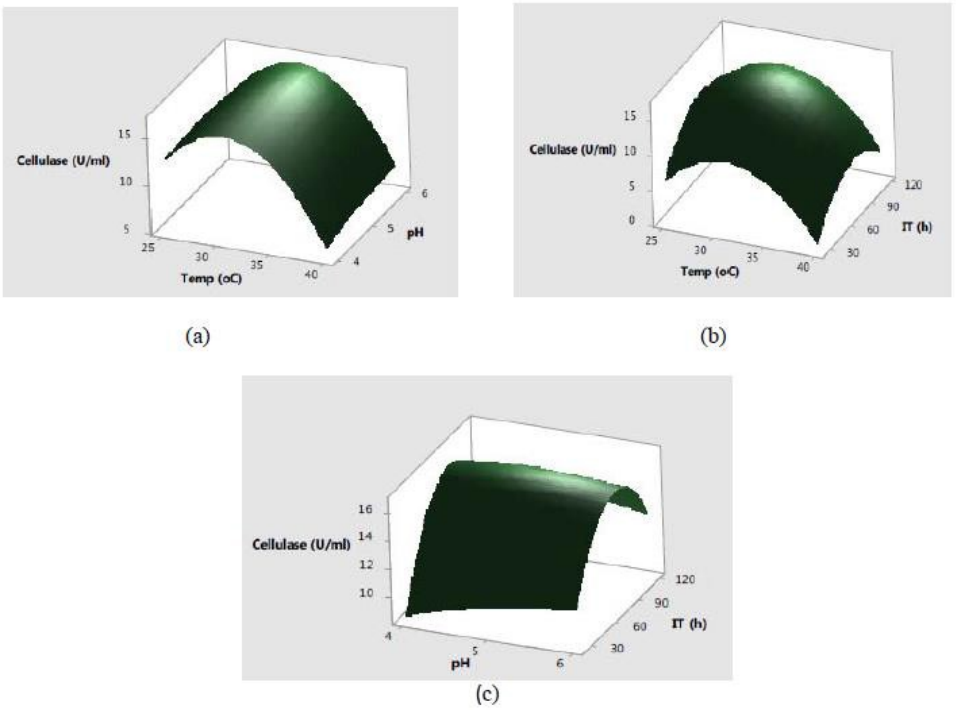


Figure 10: Response surface plots showing the effect of **(a)** temperature and pH **(b)** temperature and incubation time and **(c)** pH and incubation time on cellulase production

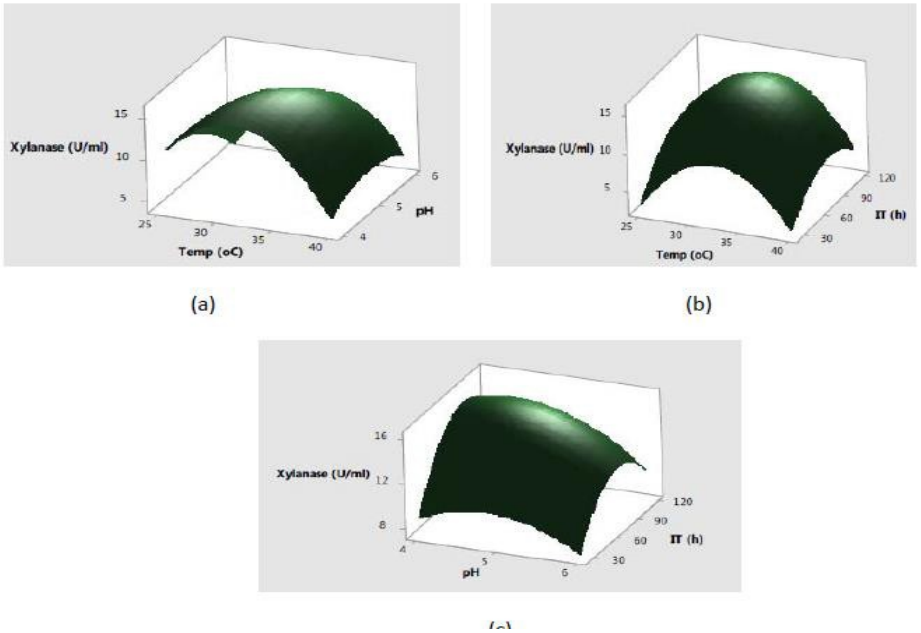


Figure 11: Interaction effects of **(a)** temperature and pH **(b)** temperature and incubation time and **(c)** pH and incubation time on xylanase production

The cumulative effect of submerged fermentation variables on laccase production are presented in **Figure 12 (a − c)**. The results in **Figure 12 (a)** indicated that the higher laccase activity was obtained at mid values of temperature with no significant effect of change in pH.

The combined effect of temperature and incubation time on laccase production (**Figure 12 (b)**) demonstrated that the maximum laccase production could be achieved at middle values of temperature and incubation time. Moreover, the significant effect of incubation time and non-significant behaviour of change of pH on laccase production was also observed in **Figure 12 (c)**.

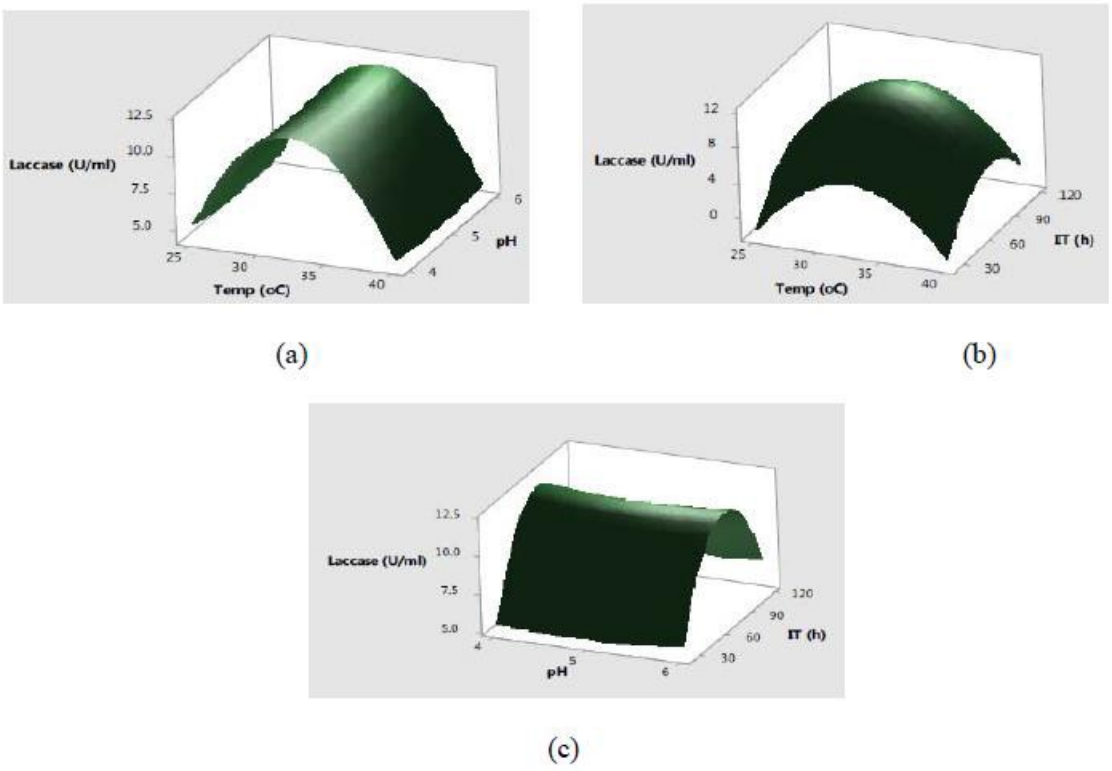


Figure 12: Effect of **(a)** temperature and pH, **(b)** temperature and incubation time and **(c)** pH and incubation time on laccase production

CONCLUSION

The optimum values of process parameters for all the three enzymes were seen to be equal to 31°C, 5.0, 77 h, and 140 rpm for temperature, pH, incubation time and agitation rate respectively. The experimental results obtained through the triplicate runs by utilizing the predicted variables gave 20.6 U/ml of cellulase activity, 17.3 U/ml of xylanase activity and 14 U/ml of laccase activity. The cellulase, xylanase and laccase yield improved about 2.3 fold, 2.1 fold and 1.4 fold respectively when compared with the cellulase, xylanase and laccase yield obtained using one factor at a time approach.

OBJECTIVE 4

2.4. Characterization of crude enzyme cocktail for lignocellulolytic enzyme activities and its application in saccharification of wheat bran for bioethanol production and dye decolorization.

The application of enzymes in different biotechnological interventions needs the characterization of enzyme at different biocatalytic parameters such as process temperature and pH. As the crude enzyme extract remained stable at moderately high temperature its role in various applications such as pulp and paper, food/ feed enzymatic hydrolysis of lignocellulosic materials can be explored as thermostability is an attractive and a necessary characteristic of an enzyme. In this objective, we also explored the ability of enzyme cocktail produced by *C. pannosa* consisting of cellulase, xylanase and laccase enzymes in efficient saccharification of lignocellulosic biomass (wheat bran) required for bioethanol production and in dye decolorization.

MATERIALS AND METHODS

Characterization of lignocellulolytic activities of crude enzyme cocktail

The cell free supernatant obtained from fermentation of YEP containing 2 % wheat bran after 96 h of incubation at 30°C, 120 rpm and pH 5.0 was precipitated with 80 % ammonium sulphate. Ammonium sulphate was slowly added to the sample kept at 4°C on a magnetic stirrer to bring final concentration to 80%. For complete precipitation, the mixture was kept at 4°C overnight with gentle stirring. The following morning the mixture was centrifuged at 15,000g for 15 min at 4°C. The pellet obtained was reconstituted in 1 mL of sodium acetate buffer. The protein content of the reconstituted sample was estimated by Lowry's method of protein estimation [33]. For crude cellulase and xylanase optimum conditions supporting maximum activity were identified by varying temperature from 25 – 70°C and pH from 3.0 – 7.0. Similarly, for crude laccase the optimum conditions supporting maximum activity were identified by varying temperature from 25 − 60°C and pH from 3.0 − 6.0.

Zymogram analysis

For zymogram analysis the crude enzymes were subjected to native-PAGE analysis. The crude enzyme preparation was mixed with protein loading buffer and separated on a discontinuous gel system (Bio-Rad Life Sciences, USA) containing 12% resolving gel and 5% stacking gel under non-denaturing conditions according to a modified method of Laemmli [34]. After PAGE, the gel was divided into two pieces: one was stained with 0.05% Coomassie brilliant blue R-250 and the other piece was subjected to activity staining.

Activity staining for cellulase and xylanase was performed by incubating the gels in 50 mM sodium acetate buffer (pH 5.0) containing 2% CMC and 2% birchwood xylan respectively for 1 h at 55°C. After incubation, both the gels were stained with 0.1% Congo red for 30 min [35]. For laccase activity determination the other half of gel was incubated in 20 mM ABTS dissolved in sodium acetate buffer of pH 5.0 at 32°C till green colour band corresponding to presence of laccase developed.

Saccharification of wheat bran for bioethanol production

Saccharification potential of fungal biomass

An Erlenmeyer flask containing wheat bran (2%) and *C. pannosa* (2.9 x 10^8 fungal spores/mL) was taken and incubated for 72 h at optimum conditions. The hydrolyzed broth was collected from flask at every 12 h interval for determination of enzyme (cellulase, xylanase and laccase) activity and glucose content to determine the optimum time at which maximum saccharification was obtained. After identifying the optimized time, a fresh flask containing 2% wheat bran and *C. pannosa* was taken and incubated at optimum conditions for the optimum time period identified above to bring about maximum saccharification, after which the contents were centrifuged and the hydrolysate obtained was subjected to fermentation by *Saccharomyces cerevisiae* MTCC 174 inoculated at the level of 0.25 optical density (OD 600nm) at 30°C and 120 rpm for 72 h. An aliquot of fermented broth from the flask was withdrawn after 72 h incubation and estimated for ethanol content.

Saccharification potential of crude enzyme cocktail

An Erlenmeyer flask containing wheat bran (2%) was subjected for hydrolysis by crude enzyme mixture at similar levels of activity units as above. The hydrolyzed broth was collected from flask at every 12 h interval upto 72 h for estimation of glucose content to determine the optimum time at which maximum saccharification was achieved. Following this, a fresh flask containing 2% wheat bran and crude enzyme mixture having enzyme units similar to that used in "Saccharification potential of fungal biomass" was subjected to hydrolysis for the optimized time identified above to bring about maximum saccharification. After the incubation period, the hydrolysate was collected and fermented with *Saccharomyces cerevisiae* MTCC 174 at the level of inoculum size equal to 0.25 optical density (OD 600nm) at 30°C and 120 rpm for 72 h. An aliquot of fermented broth from the flask was withdrawn after 72 h incubation and estimated for ethanol content.

Decolorization of synthetic dyes

Dye decolorization by fungal mycelia

For decolorization experiments, five agar plugs (6 mm diameter) of active mycelium from PDA plate were transferred aseptically into 500 mL Erlenmeyer flasks containing 100 mL of potato dextrose broth with 60 mg/L of Congo red dye (CR), bromophenol blue dye (BB), coomassie Brilliant Blue R-250 (CBB R-250) and orange G (OG) dye and incubated at 30 °C for 96 h at 120 rpm. A biotic (sterilized medium, without dye addition) and abiotic (sterilized medium containing the dye but not inoculated with the fungus) control experimental were also prepared and maintained in parallel with the decolorization experiments. Culture samples were withdrawn at defined interval of 24 h upto 96 h, centrifuged at 10,000g for 20 min at 4 °C and the supernatant obtained were used for decolorization assay. The intensity of dyes was measured at their maximum absorbance of 500 nm, 590 nm, 592 nm and 478 nm for CR, BB, CBB R-250 and orange G, respectively.

Dye decolorization by crude laccase

Stock solutions of CR, BB, CBB R-250 and OG dyes were prepared in sterilized distilled water and diluted to the required concentration 60 mg/L. The decolorization of dyes by crude enzymes was carried out in 5 mL of reaction mixture containing 0.5 mM sodium acetate buffer (pH 5.0), 0.5 U/mL of crude enzyme with either CR, BB, CBB R-250 or OG dye. The reaction tubes were incubated at 30°C for 240 min. The absorbance of the mixture at maximum wavelength was recorded at the wavelength specified above against each dye. A control test containing the same amount of a heat-denatured laccase was performed in parallel.

Decolorization efficiency (DE)

The decolorization efficiency (DE) was calculated according to the following formula: DE= (A1-A2)/A1× 100%, where A1 represented the absorbance of the control, A2 represented the absorbance of the corresponding untreated sample, and DE was the dye color removal rate.

RESULTS AND DISCUSSIONS

Characterization of lignocellulolytic activities of crude enzyme mixture

The effects of diverse temperature and pH on the activities of crude cellulase, xylanase and laccase enzyme were studied, the results of which are shown in **Figure 13, 14 and 15**. The maximum cellulase, xylanase and laccase activities were obtained at 50°C with pH of 5.0.

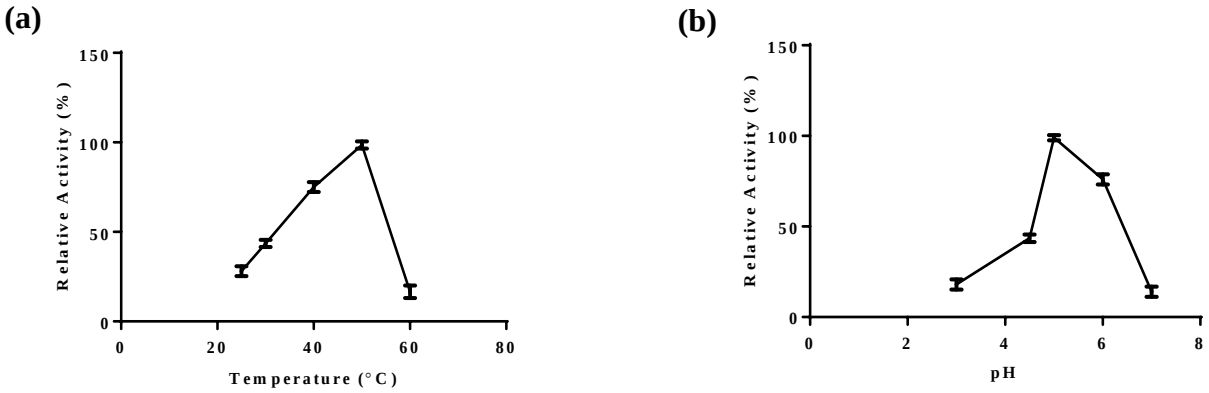


Figure 13: Effect of **(a)** temperature and **(b)** pH on activity of crude cellulase of *C. pannosa*

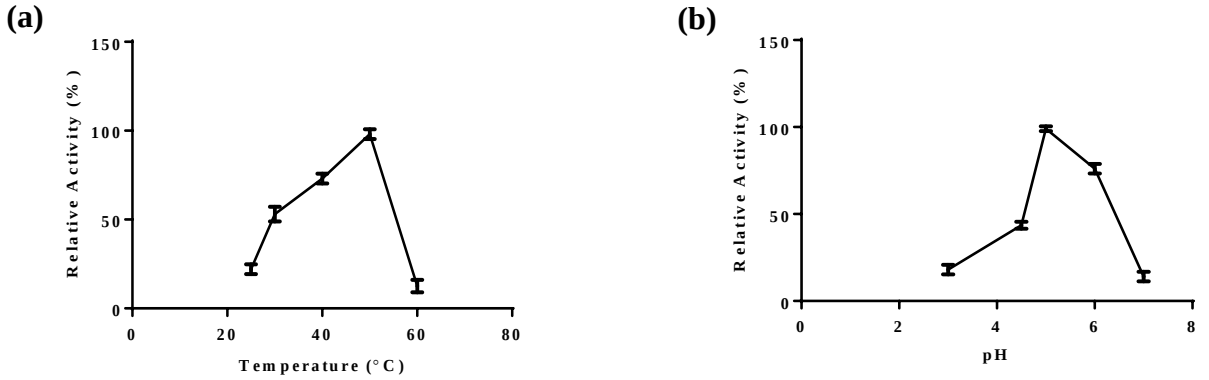


Figure 14: Effect of **(a)** temperature and **(b)** pH on activity of crude xylanase of *C. pannosa*.

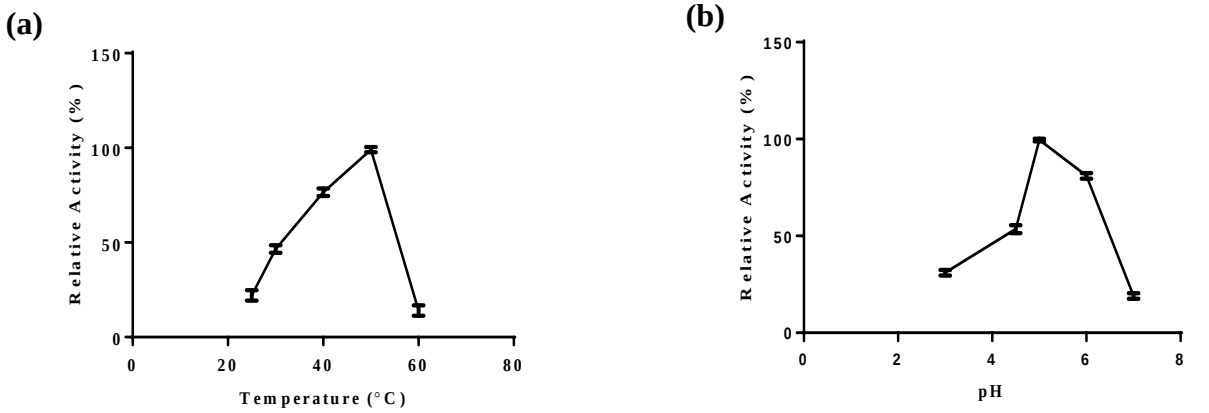


Figure 15: Effect of **(a)** temperature and **(b)** pH and on activity of crude laccase of *C. pannosa*.

Zymogram analysis

The zymogram analysis to detect the expression of cellulase, xylanase and laccase in the crude enzyme extract produced by *C. pannosa* revealed the presence of multiple cellulase isozymes (Lane 3) and xylanase isozymes (Lane 4) with the most active cellulase and xylanase being detected at ~60 kDa and ~32 kDa respectively, which appeared as a sharp clear zone against a dark red background (**Figure 16 (a)**). In case of laccase zymogram, a single ~43kDa laccase was detected which appeared as a green band against a clear background (**Figure 16 (b)**).

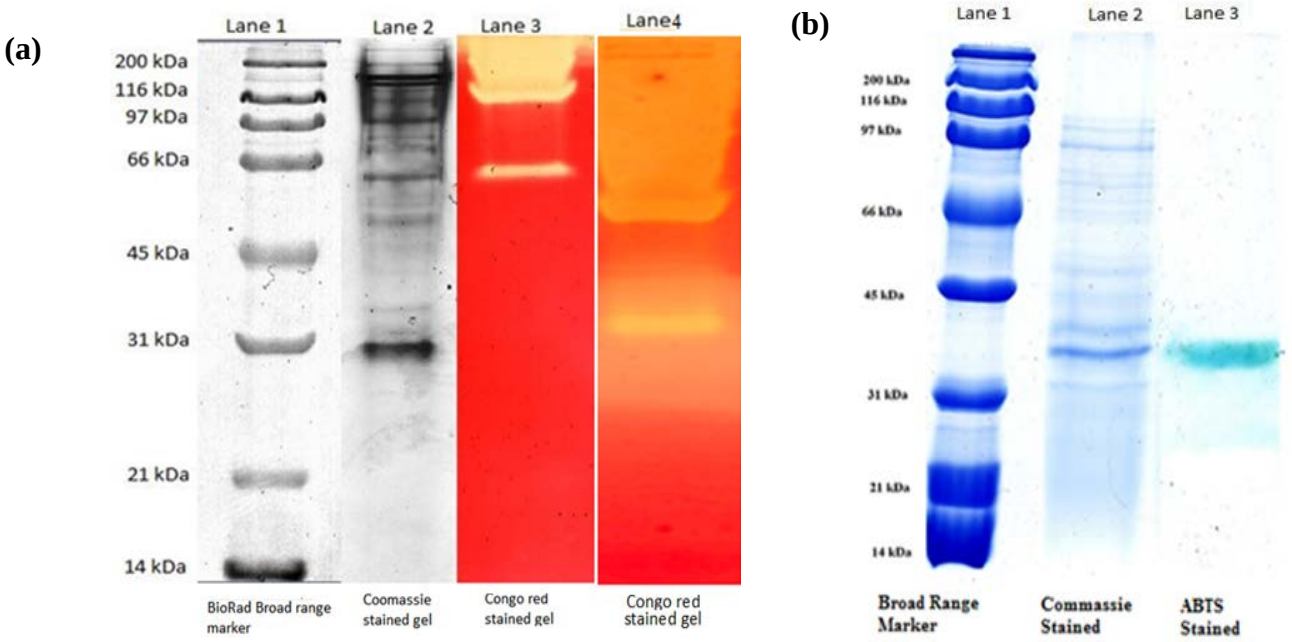


Figure 16: Zymogram analysis showing the expression of **(a)** cellulase and xylanase in the crude enzyme extract produced by *C. pannosa*. Lane 1 corresponds to protein standard with molecular weights (kDa) labelled, Lane 2 is coomassie stained sample, Lane 3 is the corresponding cellulase and Lane 4 xylanase zymogram stained with Congo red. **(b)** crude laccase from *C. pannosa.* Lane 1 corresponds to protein standard with molecular weights (kDa) labelled, Lane 2 is coomassie stained sample and Lane 3 is the corresponding laccase zymogram stained with ABTS.

Saccharification of wheat bran for bioethanol production

Assessing saccharification efficiency revealed that in the presence of fungal mycelia the flask incubated for 56 h exhibited the maximum amount of glucose (8.75 g/L) while in case of precipitated crude enzyme mixture, maximum glucose was obtained at 36 h (10.5 g/L). These flasks when subjected to fermentation by *Saccharomyces cerevisiae* gave an ethanol yield of 4.12 g/L and 4.8 g/L in case of hydrolysate obtained from flasks containing fungus and crude enzyme cocktail respectively.

Saccharification of wheat bran for bioethanol production revealed that precipitation of crude enzyme cocktail obtained from *C. pannosa* resulted in efficient saccharification and ethanol production from wheat bran as compared to that in presence of fungal mycelia. It was observed that precipitated crude enzyme cocktail produced higher amounts of glucose and ethanol in lesser time as compared to saccharification and fermentation in presence of fungal mycelia. The ethanol yield obtained in our study using wheat bran as carbon source was found to be far much better than in the earlier report of submerged fermentation of pre-treated cotton stalk with *Phanerochaete chrysosporium* with an ethanol yield of 0.027 g ethanol g^{-1} [37]. Our results are comparable to work done on *Trametes hirsuta* where an ethanol yield of 4.3 g L^{-1} is reported after 96 h cultivation using wheat bran under submerged fermentation via consolidated bioprocessing [38]. This suggests that *C. pannosa* is a suitable candidate for use in the production of ethanol from lignocellulosic biomass.

Decolorization of synthetic dyes by *C. pannosa*

The decolorization results indicate that the mechanism for decolorization by *C. pannosa* consist of a combination of biosorption by fungal mycelia and biodegradation by extracellular laccase. In case of decolorization by fungal biomass, *C. pannosa* was able to decolourize CR to a maximum extent of 94% with no decolourization of orange G after 96 h of incubation (**Figure 17**). The dye decolorization efficiency of crude laccase was 40% for CR with no decolorization of orange G. The CR being an azo dye is decolourized more rapidly than both CBB R-250 and BB which are triphenylmethane dyes. The triphenylmethane dyes are known to be resistant to enzymatic decolorization as compared to azo dyes and hence require more time for decolorization [39]. The resistant nature of orange G has also been reported by Selvam et al. (2003) where they indicated around 33% of decolorization after 9 days using similar basidiomycetes named *Thelephora* species [40]. The slow mineralization of orange G might be due to the nature of ring substituents as compared to Congo red. The most common mechanisms for dye decolorization are binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation [40]. The higher decolorization by fungal biomass indicates that the major mode of decolorization by *C. pannosa* is through adsorption of the dyes onto its cell surface. This type of decolorization has been reported to be the primary mechanism of decolorization [40] and has been reported to be superior to decolorization by enzymatic preparations [41].

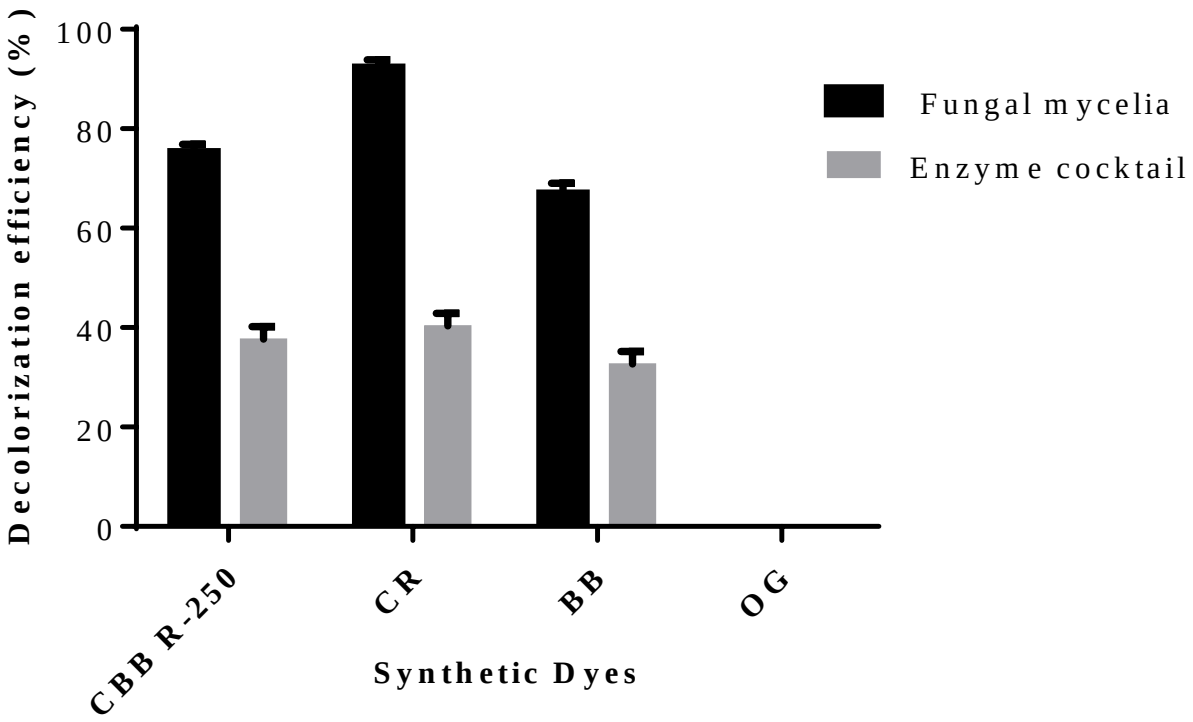


Figure 17: Decolorization efficiency by fungal mycelium and enzyme crude laccase of *C. pannosa.*

CONCLUSION

The crude cocktail was stable at 50C at a pH of 5.0. The zymogram analysis coupled with native-PAGE showed the presence of mixture of band for cellulase and xylanase while a single band for laccase was observed in *C. pannosa.* Saccharification of wheat bran for

bioethanol production in presence of fungal mycelia and by precipitated crude enzyme cocktail showed that crude enzyme cocktail is more efficient in saccharification of wheat bran in terms of duration of time required to achieve similar level of ethanol from wheat bran hydrolysate. Decolourization experiments revealed that the major mode of decolourization employed by *C. pannosa* consists of a combination of biosorption by fungal mycelia and biodegradation by extracellular laccase.

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4. PUBLICATIONS

Publication in peer reviewed journals

1. **D. Sharma**, G. Goel, S. Bansal, R. Mahajan, B. M. Sharma, and R. S. Chauhan, "Characterization of cellulolytic activities of newly isolated *Thelephora sowerbyi* from North-Western Himalayas on different lignocellulosic substrates," *J Basic Microbiol*, vol. 55, pp. 1-11, 2015.
2. **D. Sharma**, G. Goel, A. Sud, and R. S. Chauhan, "A novel laccase from newly isolated *Cotylidia pannosa* and its application in decolorization of synthetic dyes," *Biocatalysis and Agricultural Biotechnology*, vol. 4, pp. 661-666, 2015.
3. **D. Sharma**, V. K. Garlapati, and G. Goel, "Bioprocessing of wheat bran for the production of lignocellulolytic enzyme cocktail by *Cotylidia pannos*a under submerged conditions," *Bioengineered*, vol 7, pp. 88-97, 2016.

Publication in conference

1. **D. Sharma**, G. Goel, S. Bansal, R. Mahajan, B. M. Sharma, and R. S. Chauhan, Exploring white rot fungi isolated from North-Western Himalayas for lignocellulolytic enzyme activities. Presented in in international Conference on Emerging Trends in Biotechnology (ICETB-2014), JNU University, Delhi.